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PRINCIPAL INVESTIGATOR: Rosemary Foster, Ph.D.

CONTRACTING ORGANIZATION: Massachusetts General Hospital
Boston, Massachusetts 02114-2554

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Rosemary Foster, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Massachusetts General Hospital
Boston, Massachusetts 02114-2554

E-Mail: foster@helix.mgh.harvard.edu

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13. ABSTRACT (Maximum 200 Words)
The role of neurofibromin in cellular growth control is complex with available data suggesting that neurofibromin is a bifunctional modulator that may regulate proliferation by coordinating the activities of numerous molecular pathways. One important step in clarifying neurofibromin's function is identification of critical interactors whose detection and characterization may define downstream targets of neurofibromin and regulators of its activity. The purpose of our research was identification of neurofibormin-associated proteins in multiple experimental systems using high sensitivity surface enhanced laser desorption/ionization (SELDI) mass spectrometry. Our primary efforts were aimed at overcoming the longstanding difficulties of manipulating normal and mutant forms of neurofibromin in mammalian cells and our progress has been severely limited by technical difficulties that prevented controlled exogenous neurofibromin expression in target cells. We have focused on using tetracycline-inducible HSV amplicon systems to re-introduce wild type neurofibromin into neurofibromin-deficient primary mouse embryo fibroblasts and human neuroblastoma cells. Our preliminary analyses in NIH3T3 fibroblasts, neurobibromin-deficient MEFs, and human Schwann cells derived from NF1-associated tumors show tight control of tetracycline-inducible gene expression and suggest that the amplicon system will be a valuable tool in the identification of neurofibromin-associated proteins in a broad spectrum of cell types, including those that are pathologically relevant to the NF1 phenotype.

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Introduction

Neurofibromatosis type 1 (NF1) is a common human autosomal dominant disorder characterized by a complex disease phenotype that includes the development of both benign and malignant tumors of the nervous system (1). The *NF1* gene encodes the neurofibromin tumor suppressor protein with clear homology to Ras GTPase activating proteins (GAPs) (2, 3). The ability of neurofibromin to negatively regulate Ras through stimulation of GTP hydrolysis is consistent with its tumor suppressor function although considerable evidence suggests that neurofibromin has important regulatory functions distinct from its RasGAP activity and its role in cellular signal transduction and growth control is complex (4-9). One critical step in clarifying the function of neurofibromin is the identification of crucial interacting proteins whose detection and characterization may help to place neurofibromin in alternative signal transduction pathways and may define both regulators of neurofibromin activity and downstream targets of neurofibromin. Current information obtained from studies using conventional methods to detect functionally relevant neurofibromin-interacting proteins is limited, suggesting that alternative approaches to this long-standing question may prove valuable in the definition of critical interacting proteins. The purpose of our research is the identification of functionally relevant neurofibromin-associated proteins using high sensitivity surface enhanced laser desorption/ionization (SELDI) mass spectrometry in multiple experimental systems. Through these powerful analyses, we will identify neurofibromin-associated proteins in both human and mouse cells and assess the functional relevance of these interactions using mutant forms of neurofibromin. We expect that these studies will lay the groundwork for further research on neurofibromin function and will open new avenues of experimentation to clarify the complex role of neurofibromin in cellular growth control.

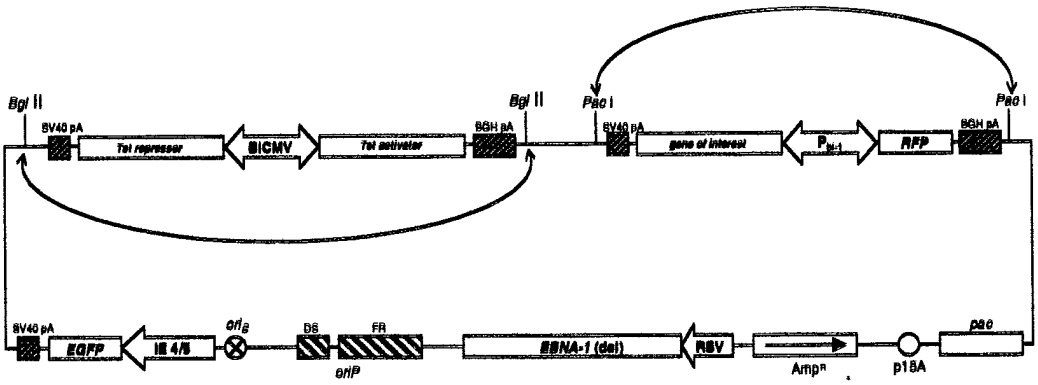
Body

Task 1. Detect neurofibromin interactors in human and mouse cells by high sensitivity SELDI

Our initial approach to the identification of neurofibromin-associated proteins in mammalian cells was simple co-immunoprecipitation analysis of neurofibromin-containing complexes isolated from neurofibromin-deficient primary mouse embryo fibroblasts (MEFs) and the human neuroblastoma cell line 90-4 following re-introduction of epitope-tagged wild type neurofibromin. The proposed experiments using SELDI to detect neurofibromin-associated proteins through co-immunoprecipitation require the use of carefully matched samples to insure that any protein detected is in fact a true associated protein and not a non-specific co-precipitating protein that is detected because of background differences used in the initial immunoprecipitation. Xandra Breakefield and colleagues in the Molecular Neurogenetics Unit have considerable expertise in the development and use of amplicon vectors that permit reproducible transgene expression in a wide variety of cell lines. Our initial studies utilized the HET2 amplicon vector shown in Figure 1A.

The amplicon vectors have proven useful in the introduction and stable expression of genes in cell lines like the neurofibromin-deficient MEFs and 90-4 that are not readily transfected through conventional methods. The amplicon vector system is designed to produce high titer helper-free virus stocks that infect target cells with high efficiency (10, 11). The vectors shown in Figure 1 allow transgene expression from a tetracycline regulatable promoter that also directs expression of a reporter (red fluorescent protein, RFP). These tetracycline-regulated amplicons are capable of high level dose-dependent induction of transgene expression in response to the application of the tetracycline analog doxycycline(M. Sena-Esteves and X. Breakefield, pers. comm.). The amplicons shown in Figure 1 also contain a tetracycline-independent reporter (enhanced green fluorescent protein, GFP) to monitor infection efficiency.

A



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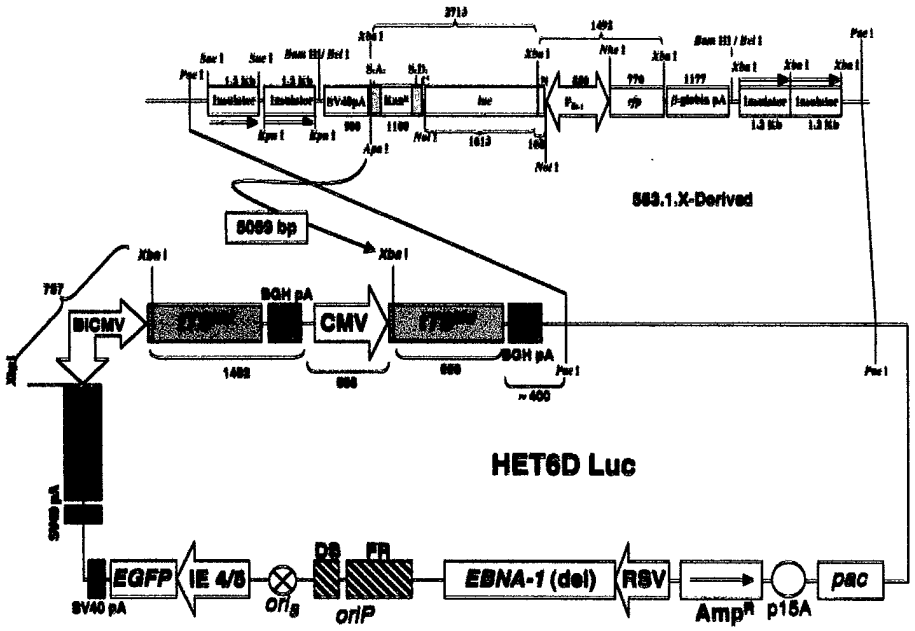

Figure 1 Tetracycline-regulated amplicons

The tetracycline-regulated amplicons HET2 (panel A) and HET6D Luc (panel B) are shown. The HSV/EBV amplicon backbone was derived from previously described constructs (10) and includes the reporter gene enhanced green fluorescent protein (EGFP, Clontech) under an immediate early viral promoter (HSV IE4/5), and the amplicon elements oriP, a latent origin of DNA replication, and a mutant version of the EBNA-1 gene. The amplicons also contain a cassette carrying the tet-silencer protein and the tet-activator protein under control of a bidirectional CMV promoter (12). In HET2, another cassette contains a multicloning site (MCS) for insertion of the gene of interest, and the reporter gene red fluorescent protein (RFP, Clontech) both of which are under the control of a bi-directional tet-responsive promoter (Clontech). HET6D Luc is derived from the HET2 vector and contains many of the same features of the amplicon backbone. The expanded region shows the elements surrounding the cloning site in which the transgene of interest replaces the luciferase coding sequence. The bidirectional promoter driving RFP and transgene expression is tetracycline-responsive.

As outlined in our midterm report, we tested the general utility of the HET2 amplicon by first analyzing the potential leakiness of the tetracycline-controlled promoter. Briefly, we infected NIH3T3 cells with the amplicon carrying no transgene and examined both GFP and RFP expression in the absence of doxycycline 48 hours post-infection. We could readily detect GFP+ NIH3T3 cells indicating efficient amplicon infection. Although we could detect some GFP+/RFP- cells, we observed strong expression of the RFP reporter protein in the absence of doxycycline suggesting a high degree of constitutive transcription from the tetracycline-dependent promoter. Because even low (2-3 fold) levels of neurofibromin overexpression can inhibit cellular proliferation (9), our studies require tight regulation of exogenous neurofibromin expression. This requirement prevented the use of the HET2 amplicon system in our studies and we turned to an improved version of the original HET2 vector which contains elements that are designed to reduce constitutive transcription from the bi-directional tetracycline-responsive promoter that drives transgene expression. This amplicon is shown in Figure 1B.

The major modification in the HET6D Luc amplicon is the presence of two copies of the *tTS*^{kid} gene which encodes a chimeric silencer protein that binds to the inducible promoter in the absence of tetracycline and blocks expression. Preliminary analysis of this amplicon indicates that the level of constitutive expression from the tetracycline-inducible promoter is significantly reduced as compared to the levels observed with the original HET2 vector (M. Sena-Esteves, pers. comm.). We obtained the HET6D Luc amplicon and infected NIH3T3 cells to test the level of constitutive reporter gene expression from the tetracycline-regulated promoter. The results are shown in Figure 2.

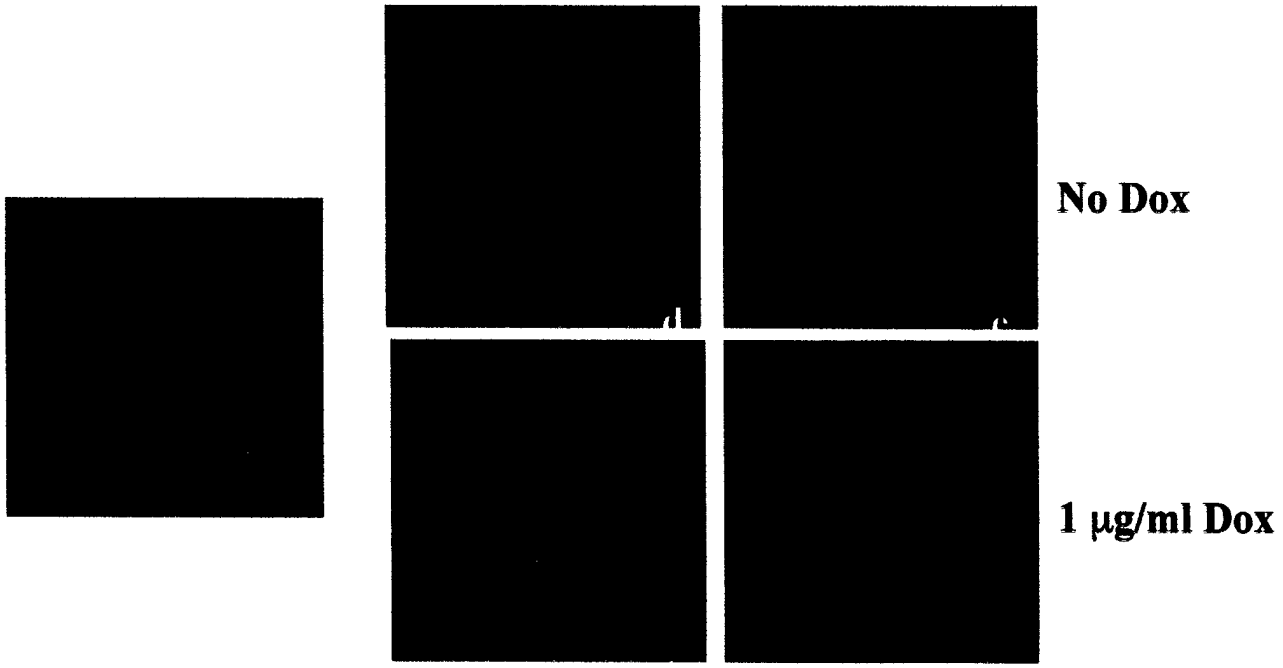

Figure 2 HET6D Luc infection of NIH3T3 cells and test of constitutive reporter gene expression
NIH3T3 cells were infected with the HET6D Luc amplicon at an MOI of 0.5. Expression of green fluorescent protein (GFP) was assessed by fluorescence microscopy 24 hours post-infection (panel a). Doxycycline was added to the cultures 24 hours post-infection and the levels of GFP and red fluorescent protein (RFP) expression were monitored at 72 hours after doxycycline addition. Panel b, GFP in the absence of doxycycline; panel c, RFP in the absence of doxycycline; panel d, GFP in the presence of 1 μg/ml doxycycline; panel e, RFP in the presence of 1 μg/ml doxycycline.

The NIH3T3 cells were readily infected by the Het6D Luc amplicon although the overall infection efficiency was low (panel a). We monitored both constitutive and inducible expression in the HET6D Luc-infected NIH3T3 cells by assessing RFP expression in the absence or presence of 1 μg/ml doxycycline at 72 hours following addition of the inducer. In the absence of doxycycline we saw no significant RFP expression in any GFP+ cells (compare panels b and c) indicating that there is little constitutive expression from the tetracycline-regulated promoter. Induction of RFP expression in the presence of doxycycline was detectable at 72 hours although only a subset of the GFP+ cells had significant levels of induced RFP expression (compare panels d and e). These preliminary results in the NIH3T3 cells were significant since it appears that there is improved control of constitutive expression in the HET6D Luc system compared to the HET2 amplicon and encouraged us to pursue studies with the improved system. The broad host range of the amplicon vector system allows us to generate a single construct that can be used for analysis in both mouse and human cells. Over the past year we have focused on testing the utility of the HET6D Luc amplicon system in both the neurofibromin-deficient MEFS and the human neuroblastoma cell line 90-4.

Our preliminary results with the HET6D Luc amplicon in NIH3T3 cells were encouraging since this modified amplicon showed relatively tight control of constitutive transcription from the tetracycline-responsive promoter in a mouse fibroblast cell line. We tested its utility in MEFs by simple infection of the neurofibromin-deficient MEF cell line 4528E. The results are shown in Figure 3.

Figure 3 Infection of neurofibromin-deficient MEFs with the HET6D Luc amplicon
The neurofibromin-deficient mouse embryo fibroblast cell line 4528E was infected with the HET6D Luc amplicon at an MOI of 0.5. Expression of green fluorescent protein was assessed by fluorescence microscopy 24 hours post-infection.

The HET6D Luc amplicon infected the 4528E MEF cell line with reasonable efficiency. We tested infected cells for induction of the RFP reporter gene using the method outlined for the NIH3T3 fibroblasts. Our preliminary experiments were inconclusive due to decreased viability of the HET6D Luc-infected MEFs over the time course of the experiment. Optimization of infection and induction conditions in the neurofibromin-deficient MEFs can be readily determined through further analysis.

Our results with the neurofibromin-deficient human neuroblastoma cell line 90-4 were disappointing as we were unable to detect even low level infection of the target cells with the HET6D Luc amplicon (data not shown). This limitation led us to consider alternative human cell lines that could be used inn the co-immunoprecipitation and SELDI analyses outlined in the original proposal. We

tested the HET6D amplicon in human Schwann cell lines derived from neurofibromas associated with NF1. Recent progress in the isolation and culturing of human Schwann cells from neurofibromas indicates that these tumors contain genetically distinct populations of Schwann cells (13). Selective culture conditions allow the separate expansion of heterozygous *NF1*+/- cells and homozygous *NF1*-/- cells although the relative proportion of each subtype within the tumor has not been determined. We have generated such cell lines from NF1-associated neurofibromas in collaboration with Dr. James Gusella in the Molecular Neurogenetics Unit. The Schwann cells used in our experiments were derived from two separate neurofibromas (T236 and T237) from a single individual. Schwann cell lines 236C and 237C were cultured under conditions that promote expansion of heterozygous *NF1* +/- cells. The cell lines 236F and 237F were cultured in the absence of forskolin which results in selective growth of *NF1* -/- Schwann cells (13). Figure 4 shows the infection of each of these lines with the HET6D Luc amplicon.

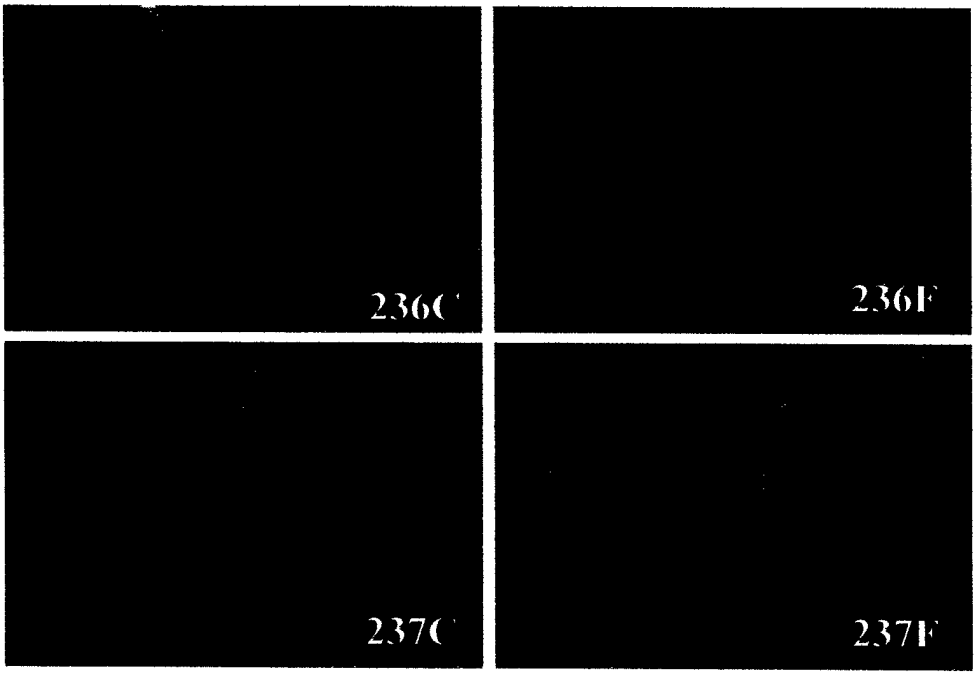

Figure 4 Infection of human Schwann cell lines with the HET6D Luc amplicon

Four separate human Schwann cell lines derived from NF1-associated neurofibromas were infected with the HET6D Luc amplicon at an MOI of 0.5. Expression of green fluorescent protein was assessed by fluorescence microscopy 24 hours post-infection.

We observed GFP+ cells in all tested Schwann cell lines although the overall infection efficiency was low. We monitored both constitutive and inducible expression in the HET6D Luc-infected 237C and 237F Schwann cells by assessing RFP expression in the absence or presence of 1 μg/ml doxycycline at 72 hours following addition of the inducer. The results are shown in Figure 5 (see next page). In the absence of doxycycline we saw no significant RFP expression in any GFP+ cells in both 237C and 237F indicating that there is little constitutive expression from the tetracycline-regulated promoter. We detected doxycycline-induced RFP expression in HET6D Luc infected 237C and 237F though only a subset of the GFP+ cells had significant levels of induced RFP expression. We obtained similar results with the 236C and 236F Schwann cell lines (data not shown). Our results with the HET6D Luc amplicon in the Schwann cell lines are significant because we can now use the amplicon to exogenously express wild-type and mutant neurofibromin in tumor derived cells. In this manner, we can identify neurofibromin interacting proteins in a pathologically relevant cell type.

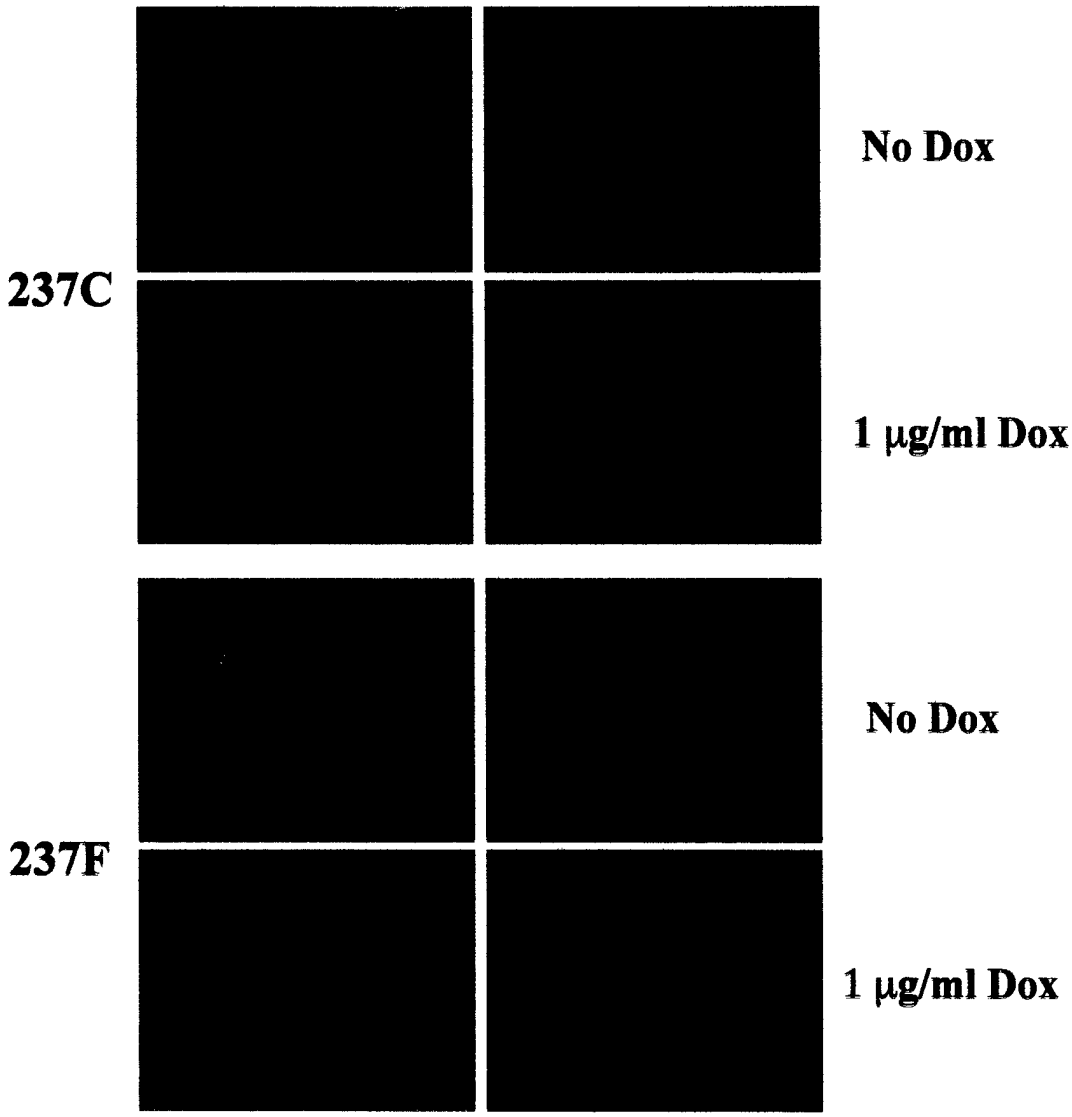

Figure 5 Induction of reporter gene expression in Het6D Luc-infected Schwann cells

The Schwann cell lines 237C and 237F were infected with the HET6D Luc amplicon at an MOI of 0.5. Doxycycline was added to the cultures 24 hours post-infection and the levels of GFP and RFP expression were assessed by fluorescence microscopy 72 hours after doxycycline addition.

Task 2. Analyze association of mutant neurofibromin proteins with identified interactors

The goal of Task 2 was to generate mutant forms of neurofibromin in the tetracycline-inducible expression vector and test the ability of each to associate with the candidate neurofibromin interactors identified in Task 1. Such analyses are one means of assessing the relevance of the identified interactors to the normal function of neurofibromin. The planned neurofibromin constructs outlined in the proposal included a series of point mutants with disease-causing amino acid substitutions in both the well-characterized GAP-related domain and a proposed second functional domain comprising exons 11-17 (8, 14). Our progress in the construction of these mutants was initially hampered by the difficulties in developing a reliable inducible expression system in the neurofibromin-deficient mouse and human cells as described in Task 1. Our encouraging results with the HET6D amplicon system in the MEF and Schwann cell lines led us to begin construction of a HET6D Luc-derived amplicon vector carrying

FLAG-tagged wild-type neurofibromin. Our progress was limited by difficulties in generating a full-length neurofibromin cDNA clone in the HET6D amplicon vector due to well-recognized toxic effects of neurofibromin expression in E. coli. We utilized a variety of cloning strategies designed to overcome these problems but have not yet generated the relevant construct for use in the initial identification of neurofibromin interactors in human and mouse cells. The generation of HET6D amplicon vectors carrying epitope-tagged mutant forms of neurofibromin has not been initiated. These vectors will be important reagents for future studies of neurofibromin function.

Key Research Accomplishments N/A

Reportable Outcomes N/A

Conclusions

Our efforts of the past year have been directed at surmounting one major obstacle to progress in NF1 research, namely the inability to manipulate normal and mutant neurofibromin expression in a relevant cell system. Our work in establishing reliable exogenous neurofibromin expression using the HET2 tetracycline-inducible amplicon system (task 1) in primary mouse embryo fibroblasts and human neuroblastoma cells has proven difficult and has limited our ability to identify cellular proteins which interact with neurofibromin. The limitations of the HET2 amplicon system also prevented us from initiating studies of the association between candidate interacting proteins and neurofibromin mutants harboring disease-causing alterations (task 2). These difficulties were overcome in part through the utilization of the HET6D Luc vector which contains elements designed to reduce constitutive transcription from the bi-directional tetracycline-responsive promoter that driving transgene expression. Our analyses of tetracycline-regulated reporter gene expression in HET6D Luc-infected NIH3T3 cells and human Schwann cells derived from NF1-associated tumors showed tight control of expression. These results suggest that this amplicon system will be a valuable tool in the identification of neurofibromin-associated proteins in a broad spectrum of cell types, including those that are pathologically relevant to the NF1 disease phenotype. We expect that such studies utilizing the HET6D Luc amplicon system will define new routes of neurofibromin function and open up unexplored areas in NF1 research. Moreover, these studies will provide insight into the molecular pathology of NF1 and identify potential targets for novel therapeutic approaches and management of this disorder.

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Appendices

List of personnel

Rosemary Foster, Ph.D.
Rajani Shelke, Ph.D.
Kathleen Ervin, B.S.
Kristy Kuplast, B.S.